CYTOGENETIC EFFECTS OF CHEMOSTERILANTS IN MOSQUITOES. II. MECHANISM OF APHOLATE-INDUCED CHANGES IN FECUNDITY AND FERTILITY OF AEDES AEGYPTI (L.)

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The successful eradication of the screw-worm fly from the island of Curaçao and the southeastern United States through the release of males sterilized by gamma-radiation (Knipling, 1959) has stimulated great interest in the possibility of using certain chemicals for the same purpose. As a result, considerable progress has been made in this field during the last few years. The field of insect chemosterilants is expanding so rapidly that three reviews (Weidhaas and McDuffie, 1963; Smith, 1963; Smith *et al.*, 1964) have appeared almost simultaneously. However, a perusal of the literature indicates that most of the research in this area is skewed in the direction of applied prospects for insect control. Relatively small effort has been expended in studying some of the biological effects of chemosterilants or the detailed mechanisms by which they interfere with insect reproduction.

Smith *et al.* (1964) have prepared an extensive list of some of the more important chemosterilants used against a number of insect species. With mosquitoes, approximately 20 chemical compounds have been shown to affect the fertility of *Anopheles quadrimaculatus*, *Aedes aegypti* and *Culex tarsalis* (Weidhaas and McDuffie, 1963). However, three of them, apholate, aphamide and tepa, appear to be more promising. They have been used to sterilize both sexes of *A. quadrimaculatus* and *A. aegypti* (Weidhaas *et al.*, 1961) and *Culex quinquefasciatus* (Murray, 1963).

Some studies have been conducted to analyze the effects of chemosterilants on insect gonads. An inhibition of ovarian growth in *Drosophila melanogaster* with folic acid antagonists was reported by Goldsmith *et al.* (1948) and Goldsmith and Frank (1952). Mitlin *et al.* (1957), and Mitlin and Baroody (1958) showed similar effects in house flies treated with different mitotic poisons and tumor-inhibiting substances. Alkylating agents have been likewise shown to retard ovarian growth in house flies (LaBrecque, 1962) and screw-worm flies (Chamberlain, 1962; Crystal and LaChance, 1963). Bertram (1963) observed ovarian degeneration in *Aedes aegypti* after thiotepa treatment. Most of these studies, however, did not go into the histological basis for the decreased ovarian size. Except perhaps in *Drosophila melanogaster* (Cantwell and Henneberry, 1963) and *Musca domestica* (Morgan and LaBrecque, 1962) detailed studies of the effects of chemosterilants on insect oogenesis have not been reported.

[1] This work received support from the following sources: Research grants No. GM 11753 and AI 02753, National Institutes of Health, U. S. Public Health Service and the Radiation Laboratory operated by the University of Notre Dame and supported in part under Atomic Energy Contract (11-1)-38. This is AEC Document No. COO-38-353.

Present studies were undertaken to find a cytogenetic basis for apholate-induced changes in the fecundity and fertility of *Aedes aegypti*. The effects of apholate on the mitotic chromosomes in the brain cells of this species have been reported in an earlier paper (Rai, 1964). The effects of this chemical on the development of ovarian tissues are presented in this paper. Some observations on the effect of apholate on testicular tissues are also included.

Materials and Methods

The Rock strain of *Aedes aegypti* was used in this study. Eggs of this strain were hatched in deoxygenated water. The growing larvae were reared in white enamel pans containing tap water in an incubator maintained at 80.6° F. Two days after hatching, when most of the larvae were second instars, they were hand-picked and transferred to pint containers (50 per container). Each of these contained 250 ml. solution of 15 ppm apholate in tap water. Fifty larvae per cup were kept as controls in 250 ml. untreated tap water. The larvae were fed with approximately equal amounts of dog-food pellets (Gaines) from time to time.

Treated pupae were picked, rinsed in tap water and then kept in untreated tap water until emergence. After emergence the adults were taken out of the incubator and were maintained in a rearing room with a temperature of 80° F. (±4° F.) and relative humidity of 80% (±10%).

At regular intervals, ovaries from mosquitoes of known ages were dissected in *A. aegypti* saline (Hayes, 1953). Whole-mounts of the dissected ovaries were made either in this saline solution or in 0.5% acetocarmine. Some dissections of testes from 8–9-day-old, unmated, treated and untreated males were also made.

Photomicrographs of various developmental stages were taken with a 35 mm. Zeiss Ikon camera from temporary slides. Panatomic-X, black and white film was used.

Four- to five-day-old adults from treated and untreated batches were crossed in single pairs in different combinations. Ten to 20 single-pair crosses were set up in each case and 1–3 egg batches were collected from each female after successive blood feedings. Spermathecae of several females kept with treated males were examined for the presence of motile sperms.

Normal Ovarian Development

The normal course of oogenesis in mosquitoes has been described in considerable detail by various investigators (Mer, 1936; Parks, 1955; Bertram, 1962). Since the present account is concerned chiefly with the effects of apholate on oogenesis in *A. aegypti*, it is necessary to describe briefly the normal ovarian development. The following stages of follicular development, described by Mer (1936) for *Anopheles elatus*, may be applied to *Aedes aegypti* also:

N: Follicle consists of 8 undifferentiated cells; cubical epithelium cells surround the spherical follicle.

I: One oocyte gets differentiated from the seven nurse cells.

I–II: Some yolk appears around the nucleus in the oocyte cytoplasm; follicle becomes oval in shape.

II: Oocyte grows by the accumulation of numerous yolk granules and occupies about half the follicle.
III: Oocyte grows to about ¾ of the follicle; oocyte nucleus obscured by the yolk.
IV: Follicle elongates and the nurse cells are restricted to a small uppermost part of it; oocyte fills almost the entire follicle.
V: Chorion appears around the egg; remains of the nurse cells are found at the proximal end of the follicle; egg floats appear and the egg is ready for laying.

Results

Effect on female fecundity

Data included in Table I indicated that treating the female larvae with 15 ppm apholate solution at second instar resulted in almost complete infecundity. Most of the females emerging from larvae so treated (hereafter designated treated females) did not oviposit. Some that did, laid only a few eggs, much fewer than any of the untreated females. Although there were some exceptions, the treated females that laid some eggs after the first blood meal were usually the ones that oviposited after subsequent blood meals also, and *vice versa*.

Table I

Effect of 15 ppm apholate on the fertility and fecundity of Aedes aegypti

Cross*		1st blood meal		2nd blood meal		3rd blood meal	
Female	Male	Eggs laid/ ♀	% Eggs hatched/ ♀	Eggs laid/ ♀	% Eggs hatched/ ♀	Eggs laid/ ♀	% Eggs hatched/ ♀
U	U	109(12)**	97.7	113(11)	97.6	115(9)	96.7
U	T	100(18)	10.0	109(14)	5.7	86.5(13)	4.4
T	U	7(11)	34.0	4(9)	68.2***	5(9)	29.6
T	T	3(17)	5.6	2(15)	18.0	1(12)	0.0

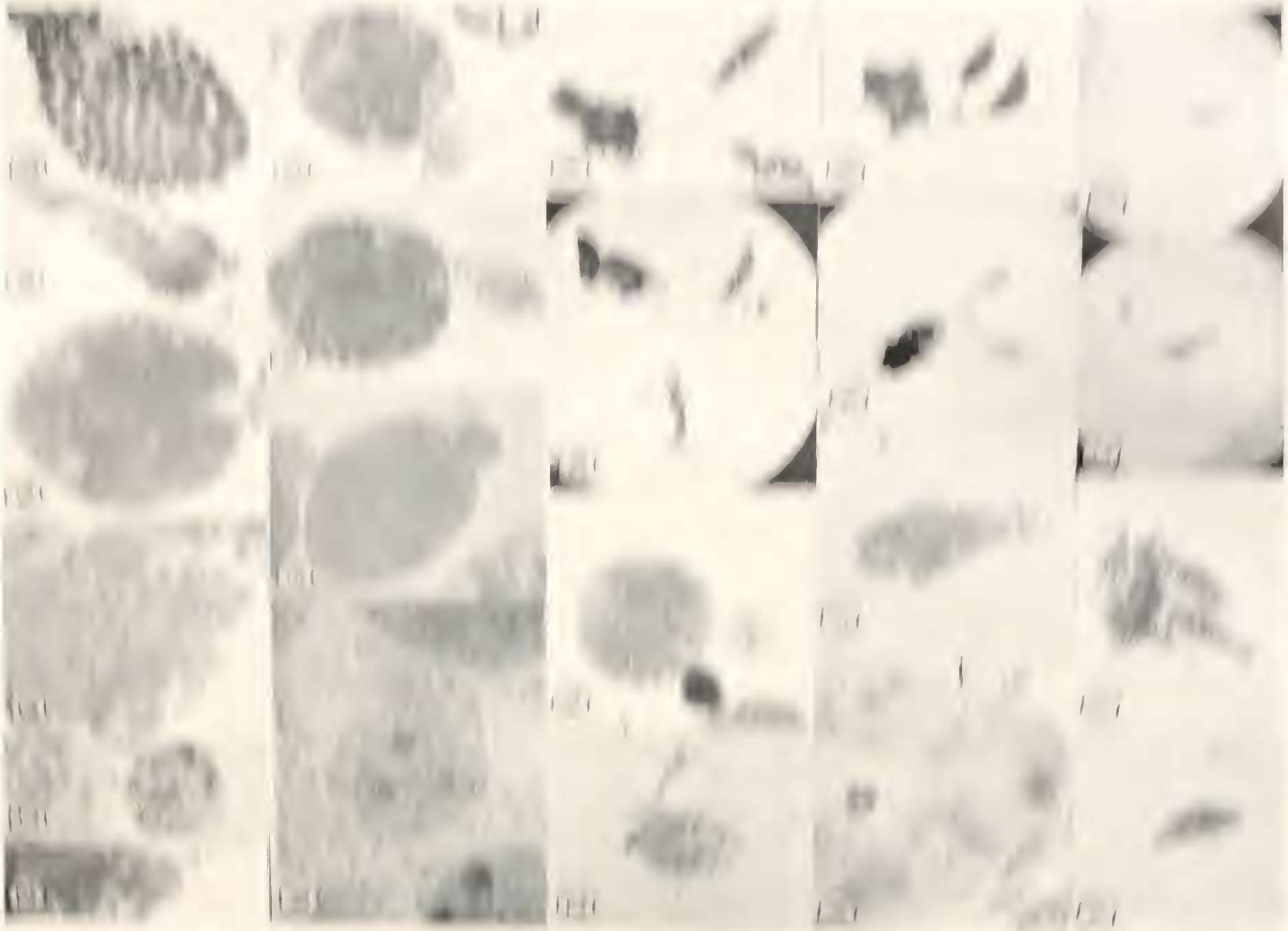
* U = untreated; T = treated.
** Figures in parentheses represent number of single pair crosses made.
*** Only one ♀ laid 39 eggs, out of which 27 hatched.

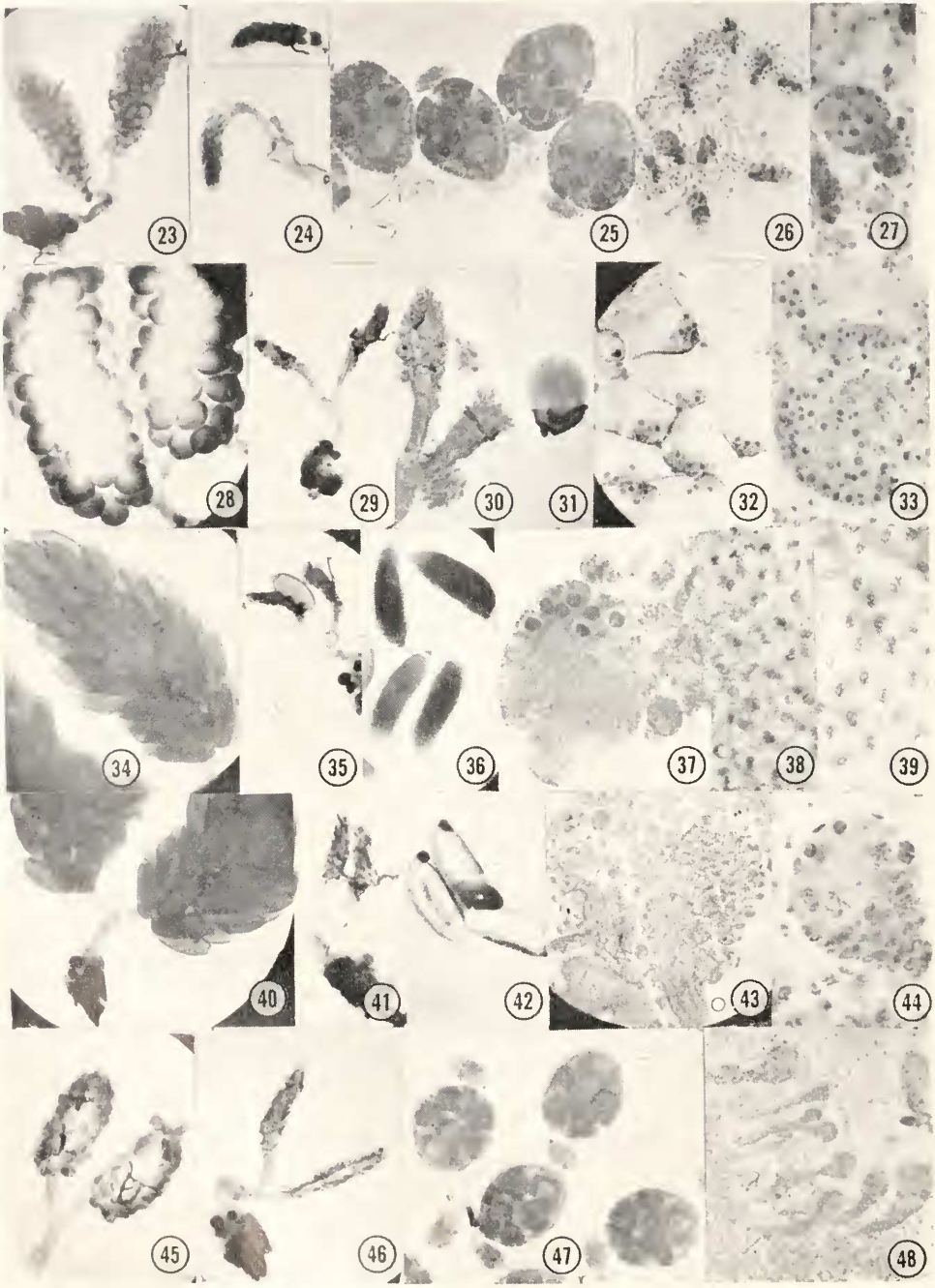
Effect on oogenesis

The details of this process in the present account will be restricted to the facts needed to evaluate properly the effects of apholate on oogenesis and to make the experimental portion of this investigation comprehensible. Results of ovarian dissections from different age groups follow. Reference made to various stages in developing follicles is based on Mer's (1936) classification.

24–28-hour-old pupae (Figs. 1–4)

There did not appear to be much difference in the size of the ovaries of the untreated (Fig. 1) and treated (Fig. 2) 24–28-hour-old pupae. The primary (first) follicle consisted of 8 undifferentiated cells with rather prominent nuclei in both the untreated (Fig. 3) and treated ovarioles (Fig. 4). This corresponds to stage "N" of Mer (1936).





FIGURES 23–27 and 33 (6 hours after blood feeding). Note the difference in size of the ovaries from control (Figure 23; 20×) batches and those of the treated ones (Figure 24; 20×). The follicles in the former are at Stage II (Figure 25; 80×); the latter show follicular

Approximately 24 hours after emergence the ovaries and the ovarioles in the controls enter a resting stage and do not develop significantly until the female is given a blood meal.

50-hour-old adults (Figs. 13–16)

Some yolk granules were clearly visible around the nucleus in the oocyte cytoplasm towards the posterior end of the primary follicle in the untreated females (Figs. 13–15; stage I–II). The yolk granules, which appear much earlier, may occupy one third of the follicle in 50- to 74-hour-old adults.

In the treated material, nurse cells in the first follicles in some cases showed signs of degeneration, as evidenced by clumps of chromatin material. At least three such clumps are visible in Figure 16.

87–91-hour-old adults (Figs. 17–22)

Ovaries in untreated mosquitoes were considerably larger and a dissection of each disclosed a large number of uniformly developing ovarioles (Fig. 17). In the treated ovaries, however, most of the ovarioles were considerably retarded in development (Figs. 18 and 20). This retardation usually occurs to different degrees in different follicles. It may depend upon the stage of follicular development at which degeneration begins. As a result, a great variability in follicle sizes was observed. A few of them approach the size of those in the controls. These may ultimately be laid as eggs. Mostly, however, they remained small and without much nuclear differentiation. Approximately one third of the developing follicles in untreated females was occupied by yolk (Fig. 19), but the oocyte nucleus was still quite visible. The follicular epithelium in controls was well defined, but showed

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degeneration to different degrees (Figure 26; 80 × and Figure 27; 200 ×). The disintegration of follicular epithelium is almost complete (Figure 33; 200 ×) in a treated female.

FIGURES 28–32 (24 hours after blood feeding). Ovaries from untreated (Figure 28; 20 ×) and treated females (Figure 29; 20 ×) differ still greatly in size. A squashed ovary from a treated female is shown in Figure 30 (20 ×). Yolk in the oocyte cytoplasm occupies most of the stage III follicle in untreated females (Figure 31; 20 ×). The nurse cells, which are pushed down towards the anterior end of the follicle, become clearly visible when a coverslip is gently put on (Figure 32; 20 ×).

FIGURES 34–39 (50 hours after blood feeding). Control ovaries are shown in Figure 34 (20 ×) and the treated in Figure 35 (20 ×). Note one well developed egg follicle in the latter. Follicles in the former are at stage IV (Figure 36; 20 ×), and are almost full of yolk. Figure 37 (80 ×) shows another stage IV follicle developing in a treated female. Neighboring follicles in this case are degenerated. The follicular epithelial nuclei in an untreated female are mostly in interphase (Figure 39; 200 ×), whereas most of those in a follicle from a treated female are pycnotic (Figure 38; 200 ×). Note the presence of minute granules around the walls of the latter.

FIGURES 40–44 (74 hours after blood meal). The follicles in control ovaries (Figure 40; 20 ×, and Figure 42; 20 ×) have developed to mature size and shape (Stage V) while the ovaries of treated mosquitoes remain much smaller (Figure 41; 20 ×). An ovarian squash shows complete follicular degeneration (Figure 43; 20 ×). One of the follicles from this squashed ovary is shown at higher magnification in Figure 44 (320 ×).

FIGURES 45–48 (5-day-old females). Ovaries of untreated females immediately after first oviposition are shown in Figure 45 (20 ×) and those of treated females of the same age in Figure 46 (20 ×). The latter did not oviposit. The ovarioles from control and treated ovaries are shown in Figure 47 (20 ×) and Figure 48 (80 ×), respectively.

various stages of degeneration in treated females (Fig. 21). A dumb-bell-shaped telophase bridge in a dividing nucleus of follicular epithelium is shown in Figure 22.

6 hours after blood feeding (Figs. 23–27, and 33)

Quick changes take place in ovarian development after blood feeding. Treated females take blood as eagerly as the untreated ones. The ovaries on the whole were much larger in the untreated (Fig. 23) than in the treated females (Fig. 24). The yolk around the distinct oocyte nucleus occupies approximately one half of the first follicle in the ovarioles in controls (Fig. 25; stage II). Most of the ovarioles in the treated females, however, were almost completely atrophied (Fig. 26). Oocytes were not obvious in these follicles (Fig. 27). The disintegration of follicular epithelium which set in some time earlier was almost completed in some follicles (Fig. 33).

24 hours after blood meal (Figs. 28–32)

The control ovaries continued to grow very rapidly (Fig. 28). However, the treated ones did not show any increase in size (Fig. 29). Whereas the ovarioles in a squashed ovary from a treated female were hardly visible (Fig. 30), the follicles in untreated females had greatly increased in size. This increase results from the aggregation of yolk granules in oocyte cytoplasm. The yolk occupied most of the follicle by this time, and the nurse cells were pushed down towards the anterior end of the follicle. The relative areas occupied by the yolk and the nurse cells stand out clearly when a coverslip is lightly put on the follicles (Fig. 32; Stage III).

50 hours after blood feeding (Figs. 34–39)

Ovaries from control batches are shown in Figure 34 and those from the treated ones in Figure 35. As mentioned earlier, the data in Table I indicated that some treated females laid some eggs. One such egg is shown developing in an ovary of a treated mosquito (Fig. 35) and another in another ovary (Fig. 37). The development of these occasional follicles proceeds as it would in an untreated female. The follicles in untreated females have assumed the shape of mature eggs (Fig. 36; Stage IV).

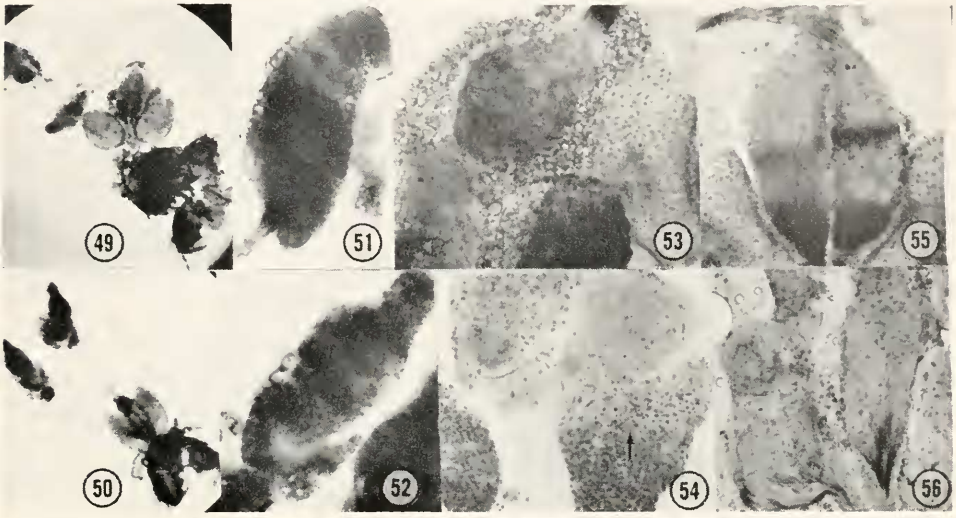
Follicular epithelial nuclei of developing eggs in the untreated series were generally in interphase (Fig. 39). In contrast, the corresponding nuclei in some of the eggs that appear to develop normally in treated mosquitoes were pycnotic and degenerated (Fig. 38). The cytoplasm, but more particularly the walls, of these cells showed the presence of minute granules not observed in the controls.

74 hours after blood feeding (Figs. 40–44)

The eggs in the untreated ovaries (Figs. 40 and 42) developed to the mature size and shape while the ovaries from the treated mosquitoes remained inhibited (Fig. 41). The ovarioles in the latter were completely inhibited in their development (Fig. 43). A typical degenerating follicle, without any epithelium, nurse cells, oocyte or yolk granules, 74 hours after blood feeding, is shown in Figure 44.

5-day-old females (Figs. 45–48)

The untreated females had laid eggs and the ovaries in these (Fig. 45) resembled those of approximately 2–3-day-old unfed females. A differentiation between the oocyte and the nurse cells was obvious in the second egg chambers (Fig. 47; Stage I–II). However, the ovaries (Fig. 46) and the ovarioles (Fig. 48) in treated females remained unchanged and degenerated.



FIGURES 49–56. Male reproductive tissues in apholate-treated and untreated males. The size of the male reproductive parts (Figure 49; 20×), including the testes (Figure 51; 80×), of 8-day-old untreated males is not markedly different from similar structures (Figure 50; 20×, and Figure 52; 80×) in 9-day-old treated males. The amount of sperm in the testes (Figure 53; 80×) and seminal vesicles (Figure 55; 80×) of the untreated males, however, appears to be more than in the testes (Figure 54; 80×) or in the seminal vesicles (Figure 56; 80×) of the treated males. The epithelium of a testis shows necrosis (Figure 54).

Effect on the male reproductive tissues (Figs. 49–56)

Dissection of the spermathecae of females caged with males developing from larvae treated at second instar (hereafter designated treated males) disclosed the presence of motile sperms. This indicated that the sperm are produced by the treated males. However, the egg hatchability data in Table I indicated that most of the sperm produced by the treated males must contain dominant lethal mutations, for when these sperms inseminated the normal females, the hatchability of the eggs was extremely low.

Several dissections of the male reproductive tissues from 8-day-old untreated and 9-day-old treated unmated, males were made. Preliminary results indicated that the size of the male reproductive structures, including the testis, was not appreciably different in the untreated (Figs. 49 and 51) and the treated (Figs. 50 and 52) males. However, the amount of sperm masses in the control testes (Fig. 53) and in the seminal vesicles (Fig. 55) often appeared to be greater than that in the same

structures (Figs. 54 and 56) in the treated males. Also, the basal portion of the testis frequently contained spermatids rather than sperms as was the case in control testes. Besides, necrosis of the epithelium of the treated testes was observed (Fig. 54). These results are in agreement with those of Cantwell and Henneberry (1963) with *Drosophila melanogaster*.

Discussion

It is obvious that rearing *Aedes aegypti* from second instar larvae until pupation in a solution of 15 ppm apholate, a commonly used alkylating agent, results in almost complete female infecundity. This effect is irreversible at least up to a month after emergence (three gonotrophic cycles). Bertram's (1963) data based on adult treatment indicated the same over a period of 6 weeks. The decreased female fecundity results from chemosterilant-induced interference with the normal course of oogenesis.

Two processes appear to be responsible for this. The young follicles are inhibited in their growth and differentiation. There is a considerable evidence that alkylating agents interfere with normal synthesis of the genetic material. Alexander (1960) has provided excellent insights into the mode of action of some of the alkylating agents. He has pointed out that the molecules of proteins, nucleic acids and different vitamins etc., have many sites to which the alkylating agents can and do attach. The sulfhydryl group (SH), the amino group (NH_2) and the acid group (COOH) are the main chemical groups with which alkylating agents react in living cells. DNA molecules may be joined together by alkylating agents in "unnatural configurations" (Alexander, p. 105). In a chromosome, if the two chromatids were cross-linked by an alkylating agent before the division of the cell, "the two could not separate properly during division and abnormalities would result" (Alexander, p. 104). This could easily lead to mitotic death. An active synthesis of chromatic material, which the nurse cells undergo during their endomitotic replications, will be thus greatly impeded. In the screw-worm fly it has been demonstrated that the greatest inhibition of ovarian growth occurs when the chemosterilants were applied during the endomitotic phase of the nurse cells (Crystal and LaChance, 1963). Also, as a direct or indirect consequence, yolk syntheses may never start in most of these follicles. It is well known that the synthesis of yolk in the oocyte cytoplasm does not start until the follicle has developed to a particular point. Besides this inhibition of follicular growth and differentiation, apholate brings about a degeneration of the developing follicles as well. This degeneration, which usually starts quite early in development, may initiate in the follicular epithelium and proceed inwards. Alternatively, the follicular degeneration also could lead to the collapse of the epithelial cells. Parks (1955) favored the former hypothesis for follicular degeneration in untreated normal females. In any case a complete breakdown of the ovarioles ensues. Cantwell and Henneberry (1963) have reported a similar degeneration of the ovarian tissues in *Drosophila melanogaster* after apholate treatment of adult flies.

Follicular degeneration is not an uncommon phenomenon in untreated, normal mosquitoes. However, in these it has been often ascribed to the ingestion of inadequate amounts of blood (Detinova, 1962) by the female. In the present study, this almost certainly was not the case.

It is interesting that in the treated females a few eggs, sometimes only one, may develop. This may be due to a number of causes. Certain follicles, whose threshold of sensitivity to chemical treatment may be lowest, might begin to develop normally. There may be critical stages in follicular development which if unaffected could permit growth and differentiation to continue until maturity. Bertram (1963, p. 333) in *Anopheles gambiae gambiae* has "some indication of a phase in early formation of the first follicles which is particularly susceptible to the action of the alkylating agent." It seems possible that a follicle beyond this stage may proceed with uninterrupted development. Furthermore, according to Detinova (1962, p. 42), follicular degeneration in normal females "is a supplementary regulatory process." In treated females this may enable some of the follicles to complete development.

A rather low hatchability of the few eggs laid by treated females inseminated by normal males (as compared with eggs from normal ♀ × normal ♂ crosses) indicates that these eggs must carry induced dominant lethals.

The effect of apholate on male reproductive tissues may be slightly different in certain regards from that on female tissues. As reported by other investigators also, the motile sperms are produced in the testes after treatment of immature stages, a treatment which in females almost stops all egg production. Furthermore, as is well known, the sperms from treated males are transferred to female spermathecae. Whereas inhibition of ovarian growth after chemosterilant treatment has been observed with a variety of different insects, results on testicular development vary considerably. Testes in adult screw-worms, treated with apholate at the larval stage, were smaller than normal size (Chamberlain, 1962). Murray (1963) got a similar effect with apholate in *Culex quinquefasciatus*. However, no marked decrease in the size of the testes in treated males was observed 6–7 days after apholate treatment in *Drosophila melanogaster* (Cantwell and Henneberry, 1963) or 9 days after treatment in the present study. It must be emphasized, however, that my results with testicular development are only preliminary at this stage.

There may be a reduction in the fecundity of males resulting from apholate treatment. This is indicated by the presence of fewer sperms in the anterior portion and mostly spermatids in the posterior portion of the testes of several treated males. Studies in sperm depletion by mating treated males to successive females would be much more definitive in this regard.

Most of the sperms produced by the treated males undoubtedly carried apholate-induced dominant lethals. This is evidenced by the failure of the eggs of untreated mothers to hatch after insemination by the treated males (Table I). It is generally believed that dominant lethals result from the induction of drastic chromosomal aberrations in the gametes. Rai (1964) has shown that apholate induces a large number of such aberrations in the brain cells of *Aedes aegypti*. It can be argued that if similar chromosomal changes are induced during gametogenesis also (there is evidence that they are), genetic disturbances would ensue in the fertilized egg. Mitotic anomalies and the resulting genetic imbalance which would result from the presence of, for example, large deletions, acentric or dicentric chromosomes or polyploid complements in a developing embryo would result in its death before embryogenesis is completed. Such fertilized eggs would not hatch. Fahmy and Fahmy (1954) have demonstrated that the failure of eggs to hatch after fertilization by chemically treated sperms in *Drosophila melanogaster* is due to disturbed cleavage

resulting from "non-eucentric chromosomal configurations." The conclusion that dominant lethality in *Aedes aegypti* results from similar mechanisms seems quite valid.

Certain limitations of the use of chemosterilants for insect control may be pointed out. Bertram (1963) has shown that treated males of *Aedes aegypti* three weeks or so after chemosterilization appear to recover from the effects and start progressively producing more normal sperms. This, as pointed out by Bertram (p. 335), may "prejudice the success of control operations based on male chemosterilization."

Field experiments with *Anopheles quadrimaculatus* (Weidhaas *et al.*, 1962) and *Aedes aegypti* (Morgan *et al.*, 1962) are not very encouraging. Studies with *A. quadrimaculatus* "in which chemosterilants were used as a biological tool, have indicated that the laboratory colony differs from the wild population in behaviour characteristics and this difference may account for the ineffectiveness of the release of males" (Weidhaas and McDuffie, 1963, p. 269).

A great deal of basic work on the diverse effects of chemosterilants on the living systems, and on the biology and behavior of the insects in natural populations must be undertaken before a proper evaluation of the potentialities of chemosterilants can be accomplished.

The author wishes to express his thanks to his colleague, Dr. Harvey A. Bender, for critical reading of the manuscript. My thanks are also due to Dr. Samuel S. Ristich of The Squibb Institute for Medical Research, New Brunswick, N. J., for providing samples of apholate used in this study, and to Mrs. Marion Ossmann for technical assistance.

Summary

1. Two-day-old larvae of *Aedes aegypti* were reared until pupation in 15 ppm apholate, a commonly used insect chemosterilant. This concentration induced almost complete female infecundity. Ovarian dissections of developing females at regular time intervals were made in order to study the underlying basis of reduced fecundity. Apholate greatly inhibited ovarian development. The follicles in treated females remained small and sooner or later underwent complete degeneration. In some of these follicles a distinction into nurse cells and oocytes never took place. In others, a complete breakdown of the follicular epithelium, nurse cells and oocyte was observed. Rarely, one or more follicles completed development in treated females. Many of these failed to hatch after insemination with normal males. This indicates the induction of dominant lethals in these eggs.

2. Motile sperms were produced by males emerging from larvae treated at second instar stage. The size of the testes in 9-day-old treated males was not greatly different from those of 8-day-old untreated males. Males used in these dissections were not mated with any females. Although conclusive data are not yet available, a lower fecundity in treated males than that of untreated males was indicated. Epithelium of the testes in some treated males showed necrosis. The so-called "male sterility" must result from induction of dominant lethality in motile sperms.

3. Some explanations concerning the mode of action of apholate and the nature of dominant lethals are discussed.

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